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An adenovirus encoding CuZnSOD protects cultured striatal neurones against glutamate toxicity

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SUPEROXIDE dismutase (SOD), a key enzyme in the detoxification of free radicals, catalyses the dismutation of superoxide $O_2^{\bullet-}$ to oxygen and hydrogen peroxide (H_2O_2). It is therefore a promising candidate for gene transfer therapy of neurological diseases in which free radicals are thought to be involved. We have constructed a recombinant adenoviral vector containing the human copper–zinc SOD cDNA. Using this vector we were able to drive the production of an active human copper–zinc SOD protein (hCuZnSOD) in various cell lines and primary cultures. Infection of striatal cells with a recombinant adenovirus expressing hCuZnSOD protected these cells from glutamate-induced cell death.

Key Words: Superoxide dismutase; Recombinant adenovirus; Striatal neurones; Glutamate; Excitotoxicity; Oxidative stress

Introduction

Accumulating evidence indicates that excessive formation of free radicals may be involved in the pathophysiology of many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis.[1-3] Oxidative stress is also implicated in acute brain disorders such as ischaemia and traumatic damage.[4] Both enzymatic and non-enzymatic systems can down-regulate the levels of oxidants in the brain.[5] Free radicals are quenched by cellular chemical scavengers (such as ascorbic acid in the cytosol or α-tocopherol in membranes), and by an enzyme pathway system consisting of superoxide dismutase (SOD) and catalase/peroxidase. SOD catalyses the dismutation of superoxide ($O_2^{\bullet-}$) to form H_2O_2, which is in turn reduced to water and molecular oxygen by catalase and glutathione peroxidase. SOD is therefore the first step in the enzyme cascade responsible for the detoxification of oxygen-derived free radicals, and increased intracellular levels of SOD may be able to protect neurones against damage by free radicals. A protective effect of endogenous SOD against oxidative damage has been reported in various studies where SOD was overexpressed in transfected cells or transgenic animals. *In vitro*, primary cultures of neurones isolated from copper–zinc SOD (CuZnSOD) transgenic mice were found to be more resistant to glutamate toxicity than controls,[6] and sympathetic neurones microinjected with CuZnSOD were partially protected against the effects of growth factor deprivation.[7] *In vivo*, transgenic mice overexpressing CuZnSOD displayed a lower vulnerability than non-transgenic controls to focal cerebral ischaemic injury,[8] MPTP- and metamphetamine-induced neurotoxicities[9,10] and cerebral infarct after cold injury.[11]

These observations suggest that gene transfer of SOD, leading to overproduction of the enzyme in neurones, may have applications for protection against several forms of neurotoxic insults. An efficient method of gene transfer into brain cells has been developed using recombinant adenoviruses, which are able to infect post-mitotic cells, and in particular neurones.[12] The adenoviral vector has also been shown to be a powerful tool for overproducing proteins of therapeutic interest in neurones *in vitro* and *in vivo*, opening the way to the development of new therapeutic strategies for neurological diseases. We have constructed an adenoviral vector carrying the human CuZnSOD gene (Ad-hCuZnSOD), to be used as a neuroprotecting tool. We report an analysis of whether infection of striatal cells with Ad-hCuZnSOD could reduce the vulnerability of these cells to glutamate-induced injury, as excitotoxicity is involved in a wide range of neurological diseases, and is linked to oxidative stress.[1]

Materials and Meth ds

Construction of recombinant adenoviruses: The production of recombinant adenovirus expressing the β-galactosidase gene was described previously,[13] and a similar strategy was used to obtain a virus expressing the human CuZnSOD gene (Ad-hCuZnSOD). Briefly, a 622 base pair hCuZnSOD cDNA (a gift from Dr Guy Rouleau, McGill University, Canada) was inserted between the *Pst*I and *Hind* III sites of a Bluescript plasmid (Stratagene) containing the polyadenylation sequence of SV40 in the *Xho*I site. The gene was then prepared as a blunt-ended *Kpn*I/*Sac*I fragment and inserted downstream from the long terminal repeat of the Rous sarcoma virus (LTR RSV) promoter in a shuttle vector containing the inverted terminal repeat (ITR) of the adenoviral genome, encapsidation sequences and adenoviral sequences allowing homologous recombination with the correct part of the viral genome (phCuZnSOD). After linearization by *Xmn*I digestion, phCuZnSOD and the large *Cla*I fragment of Ad-5 DNA were used to co-transfect the transformed human kidney cell line 293 using the calcium phosphate–DNA precipitation method. The transfected cells were overlaid with agar and plaques were screened for the presence of the recombinant adenovirus using enzyme restriction analysis and PCR. Viral stock was prepared by expansion of the recombinant adenovirus in the cell line 293 and purification by ultracentrifugation in a CsCl gradient, followed by dialysis. Virus titers were determined by plaque assays on 293 cells and expressed as plaque forming units (pfu) ml^{-1}. Ad-hCuZnSOD was obtained at a titer of 3×10^{10} pfu ml^{-1}.

Assay of hCuZnSOD enzymatic activity: Mouse NS20Y neuroblastoma cells were infected with Ad-hCuZnSOD at a multiplicity of infection (MOI) of 50 and 100 pfu $cell^{-1}$. Controls were not infected. Human CuZnSOD and host mouse CuZnSOD were identified by gel electrophoresis followed by nitroblue tetrazolium (NBT) staining. In each case a NP-40 extract was prepared from 500 000 NS20Y cells 48 h after infection, loaded on a 15% non-denaturing polyacrylamide gel, and electrophoresis was performed for 3 h at 100 V. SOD was localized by soaking the gel for 20 min in 0.3 mM NBT and 0.26 mM riboflavin, followed by immersion for 20 min in 90 mM tetramethylethylenediamine (TEMED).[14]

Cell culture and infection protocol: Striatal cultures were obtained from E15 Sprague–Dawley rat embryos (Iffa-Credo, France). The lateral ganglionic eminence was dissected out and mechanically dissociated. Cells were seeded on 24-well multiwells at a density of 250 000 cells per well in serum-free medium: DMEM containing penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}; Gibco) and supplemented with 2 mM glutamine, 100 μg ml^{-1} transferrin, 25 μg ml^{-1} insulin, 10 μg ml-1 putrescine, 5 ng ml^{-1} sodium selenite and 6.3 ng ml^{-1} progesterone (all from Sigma). More than 95% of the cells in culture were of neuronal phenotype, as assessed by cellular morphology and immunocytochemical labelling of microtubule-associated protein, β3 tubulin, 160 kDa neurofilament protein and GFAP (data not shown). Infections were performed after 3 or 4 days *in vitro* by changing culture medium for fresh medium containing the virus (Ad-βGal or Ad-hCuZnSOD) at a MOI of 100 or 300 pfu $cell^{-1}$.

Glutamate toxicity: After 6 days *in vitro*, cultures were tested for sensitivity to glutamate. A stock solution of glutamate (Na-salt, Sigma) in DMEM was added directly to the wells to a final concentration of 2.5 mM. The stock solution was prepared in DMEM. Cell viability was determined 24 h later.

Intravital staining of the culture (FDA/PI method): Cells were washed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 2.3 mM $CaCl_2$, 5.6 mM D-glucose and 8.6 mM HEPES; pH 7.4) and incubated for 5 min at 37°C with a fluorescein diacetate (FDA, 15 μg ml^{-1}) and propidium iodide (PI, 15 μg ml^{-1}) mixture. The medium was then replaced with fresh Locke's solution and cultures were immediately examined under a fluorescence microscope at 488 nm (FDA) and 514 nm (PI).[15] FDA is deesterified only in living cells to produce a green–yellow fluorescence. Neuronal injury facilitates the entry of PI into the cells, and its interaction with DNA produces a red fluorescence. Viable and injured cells were counted from three representative fields for each well (two photographs per field). The percentage of viable cells was computed by assessing the FDA/(PI+FDA) ratio for the three fields.

X-Gal cytochemistry and hCuZnSOD immunocytochemistry: To visualize transgene expression, cells were first fixed for 30 min at 4°C in phosphate buffered saline (PBS) containing 4% paraformaldehyde. β-Galactosidase was detected by incubating the cells for 2 h at 37°C in an X-Gal solution consisting of potassium ferricyanide (4 mM), potassium ferrocyanide (4 mM), MgCl2 (4 mM) and X-Gal (0.4 mg ml^{-1}, Euromedex) in PBS. For SOD immunostaining, mouse NS20Y neuroblastoma cells were incubated for 1 h with PBS containing horse non-specific serum (NSS) and 0.2% Triton X-100, and then for 48 h at 4°C with a monoclonal anti-hCuZnSOD antibody (Sigma) diluted 1/500 in PBS with goat NSS and 0.2% Triton X-100, followed by staining using the vectastain Elite ABC system (Vector laboratories). For striatal neurones, cells were incubated for 1 h in

PBS with 10% pig NSS and 0.2% Triton X-100, and then for 48 h at 4°C with a polyclonal anti-hCuZnSOD antibody (Valbiotech) diluted 1/500 in PBS containing 10% pig NSS and 0.2% Triton X-100, followed by 1 h incubation with biotinylated anti-sheep/goat Ig (1/400, Amersham) and 1 h incubation with streptavidin-biotinylated horseradish peroxidase complex (1/250, Amersham). Both mouse and rat cells were treated with VIP peroxidase substrate (Biosys, France) and hydrogen peroxide. The viability of infected cells was estimated by counting cells expressing the transgene as revealed by X-Gal staining or anti-hCuZnSOD immunochemistry in glutamate-treated and untreated cultures.

Results

A recombinant adenoviral vector expressing the hCuZnSOD gene under the control of a LTR-RSV promoter was obtained. We checked its ability to direct production of a functional enzyme. The enzymatic activity of human CuZnSOD in infected mouse NS20Y neuroblastoma cells was visualized after non-denaturing gel electrophoresis, by nitroblue tetrazolium staining. This assay is based on the generation of superoxide ions reduced by riboflavin, which converts colourless nitroblue tetrazolium into blue formazan.[14] Scavenging of $O_2^{\bullet-}$ by CuZnSOD inhibits colour development, giving rise to a colourless band at the position of the enzyme in the gel. Thus, hCuZnSOD was detected in infected cells (Fig. 1). Endogenous mouse SOD and hCuZnSOD can be discriminated by their different mobilities in the gel (Fig. 1). The band intermediate between mouse and human CuZnSOD presumably corresponds to a heterodimeric form of SOD composed of a human and a mouse subunit. The intensity of the hCuZnSOD band increased with the multiplicity of infection from 50 to 100 pfu cell^{-1}.

Human CuZnSOD was also detected by immunochemistry 48 h after infection by Ad-hCuZnSOD in NS20Y neuroblastoma cells (Fig. 2), in PC12 cells and in primary culture cells of striatum (Fig. 3C,D), cortex, cerebellum and mesencephalon (results not shown), using an antibody able to discriminate between the endogenous rodent SOD and the exogenous human form of the enzyme. The immunoreactive cells were uniformly stained, showing that the recombinant protein was present throughout the cytoplasm. The efficiency of infection with Ad-hCuZnSOD varied among the different cell types. A MOI of 300 pfu cell^{-1} for striatal neurones in primary culture led to approximately 10% of cells expressing the transgene. We investigated the neuroprotective capacities of Ad-hCuZnSOD against glutamate toxicity in striatal cells. Cells were infected with Ad-βGal or Ad-hCuZnSOD at a MOI of 100

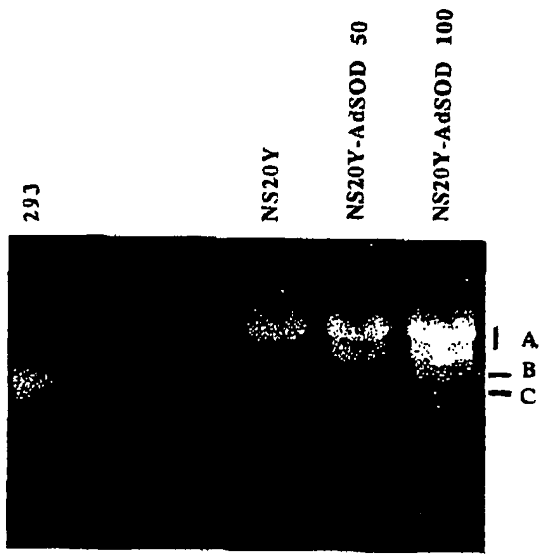

FIG. 1. Enzymatic activity of hCuZn-SOD in mouse NS20Y neuroblastoma cells non infected (lane 2) or infected with 50 and 100 pfu. cells^{-1}(lanes 3 and 4 respectively). The position of human CuZnSOD activity band is shown for uninfected human 293 cells (lane 1). (A) mouse CuZnSOD; (B) hybrid heterodimer; (C) human CuZnSOD.

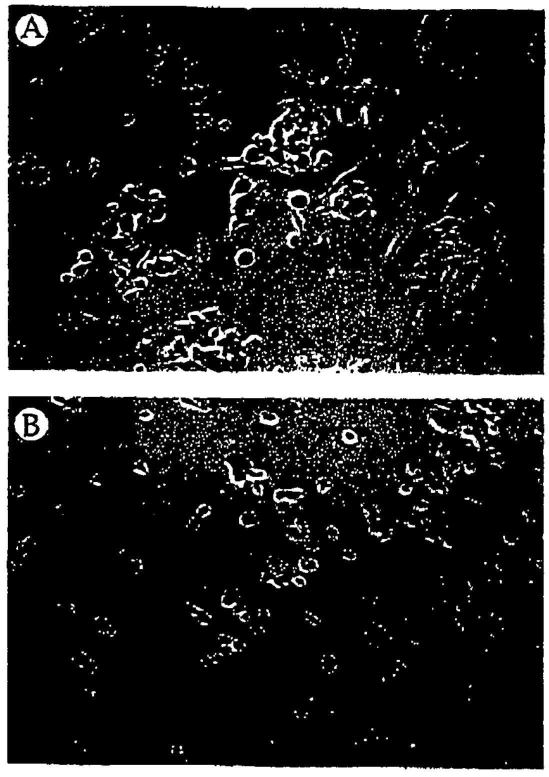

FIG. 2. Immunodetection of hCuZnSOD in cell line NS20Y. Cells were fixed for immunostaining 48 h after infection with Ad-hCuZnSOD. (A) infected cells; (B) uninfected cells.

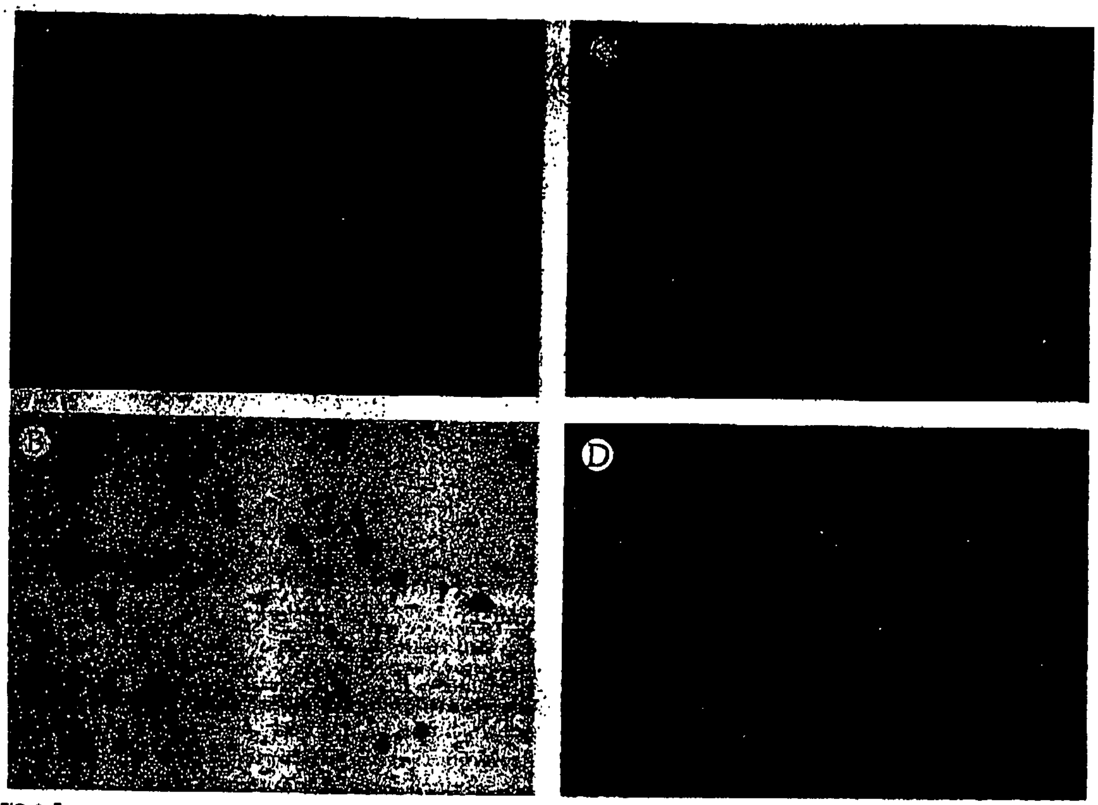

FIG. 3. Expression of transgenes in striatal neurones *in vitro*. Striatal cells were infected with Ad-βGal or Ad-hCuZnSOD at a MOI of 300 pfu cell^{-1} after 4 days *in vitro* and cells were fixed 3 days later to test transgene expression. (A) Uninfected cells (×20), (B) Ad-βGal-infected cells (×40), (C-D) Ad-hCuZnSOD infected cells (respectively ×20 and ×40).

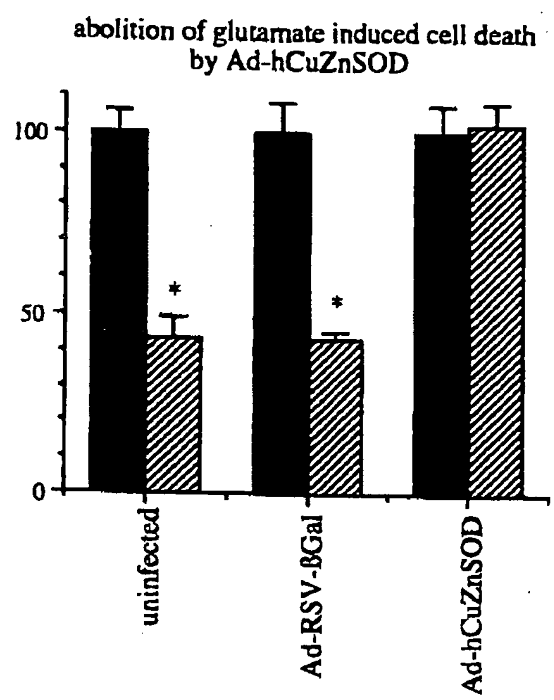

FIG. 4. Striatal neurones were infected with Ad-βGal or Ad-hCuZnSOD at a MOI of 100 pfu cell^{-1} after 4 days *in vitro*. Three days after infection, cells were exposed (▨) or not exposed (■) to 2.6 mM glutamate for 24 h and stained for β-galactosidase or hCuZnSOD. βGal and hCu2nSOD expressing cells were counted in 15 microscope fields for each well, and results were expressed as a percentage of the control value (i.e. the number of βGal and hCu2nSOD positive cells in cultures not subjected to glutamate toxicity ± s.e.m.). Triplicate sister cultures were subjected to each condition and the experiment was repeated three times. *$P < 0.002$ (student's *t*-test). Cell survival in control cultures (uninfected cells) was determined by the FDA/PI method.

and 300 pfu cell^{-1}. The expression of β-galactosidase or hCuZnSOD in striatal cells was detected by X-Gal staining or hCuZnSOD immunochemistry respectively (Fig. 3). No β-galactosidase activity was detected in Ad-hCuZnSOD-infected or control cells, and no hCuZnSOD was detected in Ad-βGal-infected or control cells (data not shown).

High doses of glutamate (2.5 mM for 24 h) applied to control primary cultures of striatum led to the death of 55% of cells, as evidenced by a decrease in fluorescein diacetate staining and a parallel increase in propidium iodide staining (Fig. 4). Infection of striatal cells with Ad-hCuZnSOD (100 pfu.cell^{-1}) conferred protection against glutamate-induced toxicity. No mortality following glutamate application was ob-

served among Ad-hCuZnSOD-infected cells, as demonstrated by counting cells immunoreactive for hCuZnSOD in glutamate-treated and untreated cultures (Fig. 4). This protective effect was due to the CuZnSOD transgene and not to viral infection *per se*: 55% of the βGal positive cells (AdβGal infected cells) died after glutamate treatment, a mortality rate indentical to that for uninfected cells in control cultures. Thus adenoviral-mediated overexpression of the hCuZnSOD gene protects striatal neurones in primary culture against glutamate-induced toxicity.

Discussion

Studies demonstrating the reduced vulnerability of hCuZnSOD transgenic mice to numerous toxic insults have indicated the neuroprotective potential of CuZnSOD. However, the use of such an enzyme to prevent neuronal death is hampered by its inability to cross cell membranes. For instance, NMDA-dependent superoxide production *in vitro* leads to neurotoxicity, and this cell death cannot be blocked by extracellular SOD.[16] Similarly, in an *in vitro* model of hypoxia (in which glutamic acid is implicated as the proximal cause of neurodegeneration), superoxide dismutase protects neurones only when taken up intracellularly under depolarizing conditions.[17] Finally, CuZnSOD delays apoptosis of sympathetic neurones deprived of growth factor, but only when the enzyme is microinjected into cells, and not when it is added to the extracellular medium.[7] These results demonstrate that CuZnSOD has to accumulate intracellularly to be able to protect injured cells. Thus, an appropriate vector is required to accomplish efficient CuZnSOD delivery to neurones. We show that adenoviral-mediated gene transfer is an efficient way to produce hCuZnSOD in neuronal cells. The exogenous enzyme is functional, as shown by activity tests in non-denaturating polyacrylamide gel. Furthermore, the intracellular levels of CuZnSOD are sufficient to protect neurones from glutamate-induced cell death.

Glutamate neurotoxicity can be mediated by the activation of glutamate receptors, or by the inhibition of cystine uptake (through the cystine/glutamate antiporter system): both mechanisms lead to the formation of free radicals. The stimulation of both ionotropic and metabotropic receptors is known to induce a rise in the cytosolic concentration of calcium which may enhance the production of free radicals by the activation of (1) a phospholipase yielding superoxide by the release and subsequent metabolism of arachidonic acid and (2) a protease which in turn accelerates the conversion of xanthine deshydrogenase to xanthine oxidase, a cellular source of superoxide.[18] Moreover, the activation of glutamate receptors may also trigger nitric oxide (NO) production,[19] yielding the peroxynitrite radical through its reaction with superoxide. Glutamate toxicity involving inhibition of cystine uptake, leading to glutathione depletion and free radical generation, has also been described in neuronal cell lines[20,21] and in cells in primary culture[22].

Independent of the possible mechanisms of glutamate toxicity, the glutamate-mediated production of free radicals is prevented in striatal cells infected by Ad-hCuZnSOD. This observation has two implications. First it suggests that in the striatal neurone model we used, superoxide radicals are involved in glutamate neurotoxicity, as they are in cerebellar[16] and cortical cells.[6] Second, our data show that Ad-hCuZnSOD is a potential therapeutic tool to prevent neurodegeneration associated with glutamate neurotoxicity. Interestingly, a recent report published during the preparation of this paper showed that Ad-hCuZnSOD could protect sympathetic neurones against NGF deprivation-induced death *in vitro*.[23] Thus Ad-hCuZnSOD displays a neuroprotective effect against both glutamate toxicity and growth factor-dependent neuronal death. Adenovirus-mediated hCuZnSOD gene transfer may therefore have therapeutic applications for a wide range of neurodegenerative disorders.

Conclusion

A high dose of glutamate applied to striatal neurones in primary culture leads to a cell death rate of 55% due to glutamate-induced oxidative stress. A recombinant adenovirus encoding hCuZnSOD is able to direct the production of this enzyme in striatal cells, and thereby to protect them against glutamate toxicity by detoxifying free radicals.

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